

Isozyme markers associated with O₃ tolerance indicate shift in genetic structure of ponderosa and Jeffrey pine in Sequoia National Park, California

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Genetic variation in isozyme markers associated with ozone tolerance differed between parental trees and their progeny in two closely related species of yellow pine.

Abstract

Effects of canopy ozone (O₃) exposure and signatures of genetic structure using isozyme markers associated with O₃ tolerance were analyzed in ~20-, ~80-, and >200-yr-old ponderosa (*Pinus ponderosa* Dougl. ex Laws.) and Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) in Sequoia National Park, California. For both species, the number of alleles and genotypes per loci was higher in parental trees relative to saplings. In ponderosa pine, the heterozygosity value increased, and the fixation index indicated reduction of homozygosity with increasing tree age class. The opposite tendencies were observed for Jeffrey pine. Utilizing canopy attributes known to be responsive to O₃ exposure, ponderosa pine was more symptomatic than Jeffrey pine, and saplings were more symptomatic than old growth trees. We suggest that these trends are related to differing sensitivity of the two species to O₃ exposure, and to higher O₃ exposures and drought stress that younger trees may have experienced during germination and establishment.

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1. Introduction

Sequoia National Park (SNP) is the most polluted national park in the U.S.A.: “In the past six years, Sequoia and Kings Canyon has had the most days exceeding the national health standard for ozone of any unit in the National Park System.¹” The source of the pollution is long distance transport of

secondary products of fossil fuel combustion into the park from both San Francisco and the San Joaquin Valley (Bytner-owicz et al., 2002). The 12-h average of ozone (O₃) in Sequoia National Park was 63 ppb over the 5-month growing season (May–September) from 1987 through 2003 (National Park Service Air Resources Division database). Over that time period, air quality in the park exceeded the state standard 32 days per year on average (Fig. 1).

The onset of poor air quality in Sequoia National Park is unknown, but possible levels of O₃ exposure can be inferred from increases in upwind population (by county), assumptions in the relationships between population and fossil fuel consumption, and constructed long term O₃ concentrations from other locations. Fossil fuel consumption was correlated with population increase in California, until more stringent air

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¹ National Parks Conservation Association report, <http://www.npca.org/turningpoint/>; and National Park Service Air Resources Division air quality database for ozone, <http://www2.nature.nps.gov/air/Monitoring/network.cfm#data.html>. Subsequent references to this database will be cited as the NPS ARD air quality database.

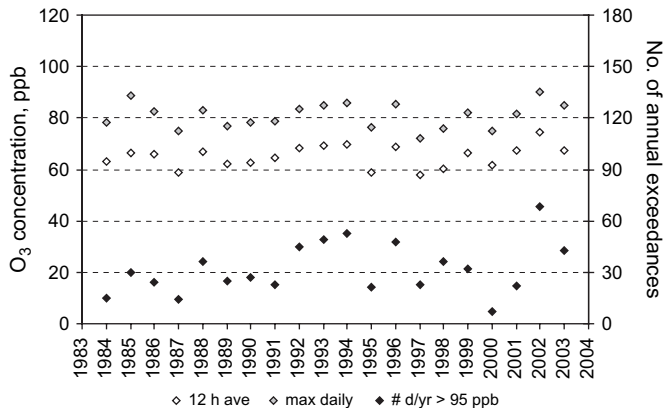


Fig. 1. Daily O_3 concentrations for 12 h (open diamonds), maximum daily O_3 concentrations (gray diamonds), and number of exceedances of California state air quality standards (black diamonds) over the 5-month period, May through September. Data were provided from the National Park Service Air Quality Division.

quality standards were achieved in the mid 1990s (Fig. 2). The longest record of O_3 concentrations were constructed (correcting for older methodologies) for a site in the western San Bernardino Mountains in the Transverse Range (Lee et al., 2003) subject to downwind transport of pollutants from Los Angeles, CA. Lee et al.'s (2003) analysis suggests a steady increase in O_3 concentrations from 1963 to peak values in the late 1970s and early 1980s, followed by a decrease in O_3 concentration by 1999 to 1960 levels. The rate of human population increase in the counties upwind differed between the southern Sierra Nevada and the Transverse Range (Fig. 3). In the Transverse Range, the first foliar symptoms at ambient (elevated) O_3 concentrations were observed in 1953 in the Transverse Range (Asher 1956, as seen in Tingey et al., 2004), with a population of ~ 5 million people in Los Angeles

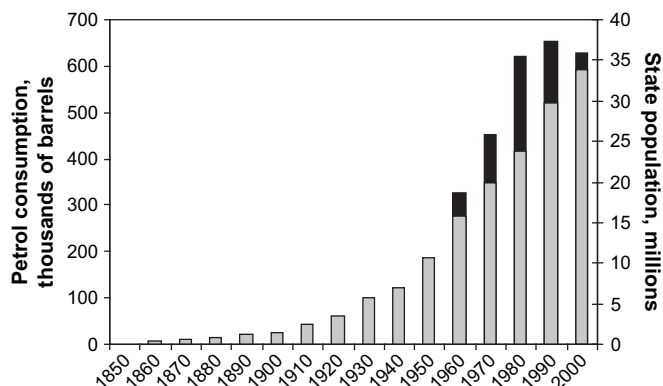


Fig. 2. California state population (2000: <http://countingcalifornia.cdlib.org/>; 1990: <http://censtats.census.gov/>; ≤ 1980 : <http://factfinder.census.gov/>) and annual petroleum consumption (<http://www.eia.doe.gov/>) in decadal increments. The apparent reduction in petrol consumption (per human) declined in 2000 relative to 1990. Consumption of other fossil fuels (e.g., natural gas and coal) had consistent consumption rates from 1964 through 1988 (2000 billion cubic feet and 2000 short tons, respectively; <http://www.eia.doe.gov/>). Then, natural gas consumption doubled until the mid 1990s, and so were less related to increases in state population.

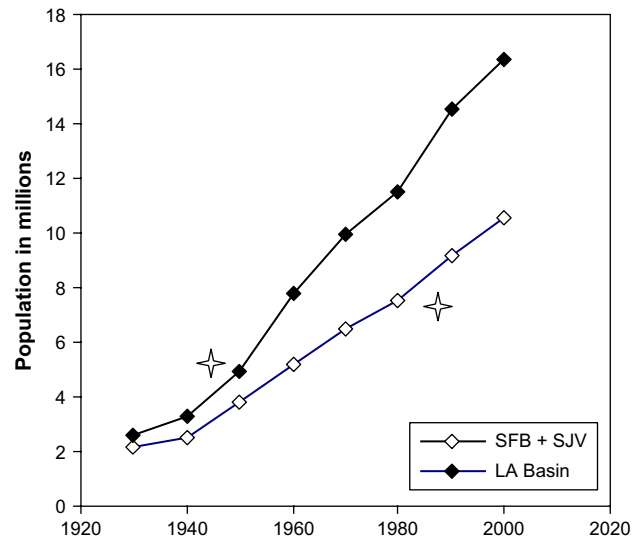


Fig. 3. Comparison of population increase of Los Angeles (LA Basin) and the San Francisco Bay and San Joaquin Valley (SFB + SJV; see website sources provided in Fig. 2). Stars indicate the year of the first foliar O_3 symptoms in the San Bernardino Mountains downwind from LA air basin (in 1961; Miller and McBride, 1975), and in 1983 in Giant Forest, Sequoia National Park downwind from SFB + SJV (in 1983; Miller et al., 1995).

metropolitan area. This population corresponded to an estimated annual fossil fuel consumption of $\sim 80,000$ barrels of petroleum (projected from Fig. 2), with emission transport ~ 100 km. In Sequoia National Park, the first foliar symptoms at ambient (elevated) O_3 concentrations were observed in 1983 in Sequoia National Park (Miller et al., 1995), with a population of ~ 8 million people in San Francisco Bay and San Joaquin Valley combined. This population corresponded to an estimated annual fossil fuel consumption of $\sim 140,000$ barrels of petroleum, with emission transport ~ 290 km. We can say with some measure of confidence that the ~ 20 -yr-old saplings in this study germinated and established in a moderately high O_3 concentration environment, the ~ 80 - and >200 -yr-old mature trees germinated and established in a low O_3 concentration environment (1914–1921 on average for mature trees), and old growth trees were established prior to industrialization.

In the southern Sierra Nevada, reduced basal area growth was observed in mature ponderosa ("PP," *Pinus ponderosa* Dougl. ex Laws.) and Jeffrey pine ("JP," *Pinus jeffreyi* Grev. & Balf.), both species of yellow pine, and was associated with moderately high O_3 exposures over the previous several decades (Peterson et al., 1987, 1991). Foliar O_3 injury of mature trees of PP, JP (Duriscoe, 1990), lodgepole pine (*Pinus contorta* Dougl. ex. Loud.), sugar pine (*Pinus lambertiana* Dougl.), and seedlings of giant sequoia (*Sequoiadendron giganteum* Bucholz.) (Miller et al., 1995) occurs under current ambient O_3 concentrations in Sequoia National Park. In southern California, basal area growth (Arbaugh et al., 1999) and simulated whole tree biomass (Tingey et al., 2004) of PP increased when air quality improved due to more stringent air quality standards.

Ponderosa pine has been previously identified as the most sensitive western conifer to elevated O_3 exposure (Miller

et al., 1983). Although closely related, PP and JP differ in sensitivity to oxidant pollution. Stomatal conductance of JP is generally 10–25% lower than that of PP, implying lower O₃ uptake (N. Grulke, unpublished data). Jeffrey pine also has greater needle concentrations of antioxidants, so that the O₃ that does enter the needle is more efficiently detoxified (M. Tausz, unpublished data).

Greater foliar O₃ injury of both PP and JP is associated with lower net plant carbon balance. The net carbon balance of both PP (Grulke et al., 2002; Coyne and Bingham, 1982) and JP (Patterson and Rundel, 1989) is significantly reduced by O₃ exposure. Ozone exposure can also significantly alter within-plant carbon allocation (Grulke et al., 2001; Grulke and Balduman, 1999), rendering younger tree age classes more susceptible to other environmental stressors such as drought and pathogens (Miller et al., 1982).

Environmental stress can affect the genetic structure of populations in different ways, e.g., through changes in the frequency of alleles and genotypes due to viability and/or fertility selection (Müller-Starck, 1985; Bergmann and Scholz, 1987; Ruetz and Bergmann, 1989), or via changes in genetic variation (heterozygosity, allele and genotype polymorphism) at the individual and population level (Müller-Starck, 1985, 1989; Bergmann and Scholz, 1989; Geburek et al., 1987; Oleksyn et al., 1994). Previous research on PP and other conifers documented significant shifts in genetic structure in response to either O₃ sensitivity (Staszak et al., 2004) or to elevated O₃ exposure (Bergmann and Scholz, 1989).

Because extant saplings germinated and established in an elevated oxidant pollution environment in Sequoia National Park, and previous generations did not, we hypothesized that the genetic structure of progeny might differ from that of parental trees in isozyme markers associated with O₃ tolerance. We tested for differences in genetic structure of seven enzyme systems and expression of canopy O₃ injury among ~20-, ~80-, and >200-yr-old PP and JP trees. This paper contributes to an understanding of the process of genetic change in populations exposed to O₃.

2. Materials and methods

2.1. Research site

The study site was located on the western slope of the south-central Sierra Nevada Mountains, on the southern edge of Giant Forest, in Sequoia National Park, California (36.6°N, 118.73°W; 1905 m). The mid-elevation forest (Sierra Nevada mixed conifer zone, *sensu* Barbour, 1988) was dominated by both PP and JP trees in a mixed age stand with white fir (*Abies concolor* (Gord. & Glend.) Lindl.), incense cedar (*Libocedrus decurrens* Torr.), California black oak (*Quercus kelloggii* Newb.), and sugar pine (*P. lambertiana* Dougl.) in the area studied. Trees were sampled within a 5 km long transect, 500 m wide, following the 1900 m topographic line (±40 m) from Sunset Rocks to just south of the road access to Crescent Meadow. The long term air quality monitoring station is located within the belt transect (Fig. 1).

2.2. Collection of plant material

Thirty-five trees in each of three age classes for both species were sampled, targeting selection of saplings (S, ~20-yr-old); mature (M, ~80-yr-old); and old growth (O, >200-yr-old), for a total of ~210 trees. Dormant lateral buds

(3–5 per tree) were collected from each tree on the southwestern aspect in November 2000 for isozyme analysis. Buds were placed in cryovials and transported and stored in liquid nitrogen (without buffer) until processed. For each tree, basal diameter (for saplings) or diameter at 1.5 m (for mature and old trees) was measured, and approximate age was determined from cambium tissue to the center of the bole at 20× magnification from an increment core. Assessment of foliar injury was made in August 2001 and 2003, a typical time for canopy health assessment. The canopy assessment consisted of counting the number of live needle age classes retained on the branch, estimating the level of chlorotic mottling on 2- and 4-yr-old needles, and measuring within-whorl needle retention, branchlet diameter, and needle length of the 2-yr-old whorl on five mid-canopy secondary branches, 3–4 branches back on mid-canopy, primary branches. On each tree, branches for foliar symptoms were sampled on all aspects. These attributes were effective in assessing the level of O₃ injury in PP (Grulke and Lee, 1997) and JP (Grulke et al., 2003). A Forest Pest Management (FPM) score, an additional O₃ injury scoring system, was assigned to each tree on the basis of the first needle age class that exhibited >15% chlorotic mottle over 30% of the needles in that whorl (Pronos and Vogler, 1981). This assessment was concurrent with the multi-attribute analysis above.

2.3. Isozyme analyses

Bud tissue of each tree was placed in a mortar and homogenized with the extraction buffer in liquid nitrogen and stored at –80 °C until analyzed. The extraction buffer was composed of: 0.1 M Tris–HCl pH 8.0 (100 ml), PVP-40 (10 g), sucrose (10 g), EDTA-2Na (0.17), dithiothreitol DTT (0.15 g), ascorbic acid (0.02 g), bovine albumin (0.10 g), NAD (0.05 g), NADP (0.035 g), and pyridoxal-5-phosphate (0.005 g). Although over 30 enzymes were assayed, little to no variation was observed in 23 of the enzymes between the two species. In the remaining seven enzyme systems, eight loci in JP and eight loci in PP varied sufficiently: fluorescent esterase (FEST, 3.1.1.1), glutamate–oxaloacetate transaminase (GOT, 2.6.1.1), malate dehydrogenase – two loci (MDH, 1.1.1.37), menadione reductase (MNR, 1.6.99.2), phosphoglucosyltransferase (PGM, 2.7.5.1), shikimate dehydrogenase (SHDH, 1.1.1.25), and triose phosphate isomerase (TPI, 5.3.1.1), using the techniques of starch gel electrophoresis (Conkle et al., 1982).

For each tree age class of each species, gene and genotype frequency, mean number of alleles (*A/L*) and genotypes (*G/L*) per locus, observed heterozygosity (*H_o*), expected heterozygosity (*H_e*), genotype polymorphism index (*P_g*), and fixation index (*F*) (Jain and Workman, 1967; Nei and Roychoudhury, 1974; Kahler et al., 1980) were calculated using the GEN computer program (Yeh et al., 1999). Allele and genotype frequencies for the compared groups of trees were evaluated using a homogeneity chi-square test, assuming equal frequency in both groups (Elandt-Johnson, 1971). Statistical significance is reported here at the *p* ≤ 0.05 level.

3. Results

3.1. Allele and genotype frequencies

For both PP and JP, the mean number of alleles and genotypes increased with increasing tree age (Table 1). The mean number of alleles per locus for PP was 2.125, 2.500, and 2.500 for saplings, mature, and old growth trees, respectively. The mean number of genotypes per locus for PP was 3.000, 3.250, and 3.125 for

Table 1
Mean number of alleles and genotypes per locus for the three tree age classes of ponderosa and Jeffrey pine (O, old growth; M, mature; S, saplings)

| Species | Alleles | | | Genotypes | | |
|----------------|---------|-------|-------|-----------|-------|-------|
| | S | M | O | S | M | O |
| Ponderosa pine | 2.125 | 2.500 | 2.500 | 3.000 | 3.250 | 3.125 |
| Jeffrey pine | 2.000 | 2.250 | 2.375 | 2.500 | 2.875 | 3.000 |

saplings, mature, and old growth trees, respectively. Similar tendencies were noted for JP: 2.000, 2.250 and 2.375 alleles per locus, and 2.500, 2.875 and 3.000 genotypes per locus for saplings, mature, and old growth trees, respectively.

In saplings, mature, and old growth trees of PP, there were 17, 20, and 20 alleles, and 24, 26 and 25 genotypes, respectively. The same tendency was found for JP: in saplings, mature, and old growth trees, there were 16, 18, and 19 alleles and 20, 23 and 24 genotypes, respectively.

Differences in allele frequency between the age classes of PP trees were significant for allele *SHDH-1*, *SHDH-2* and in the frequency of genotypes for genotypes *SHDH-11*, *SHDH-22* and *MDH3-12* (Table 2). For JP, significant differences in the allele frequency among the tree age classes were found for alleles *FEST-2*, *SHDH-2*, *SHDH-3*, *TPI-1* and *TPI-2* and in genotype frequency for genotype *TPI-11* (Table 3).

3.2. Heterozygosity

The observed (H_o) and expected heterozygosity (H_e) for the three tree age classes of PP and JP are shown in Tables 4 and 5. For PP, the average observed heterozygosity for all studied loci was higher in the older trees by 24%. The reverse tendency was noted for JP with higher observed heterozygosity in younger trees compared to old growth by 34%. The most distinct differences in observed heterozygosity for the three age classes of PP were found for loci *TPI* and *MDH5*, and for JP, loci *FEST*, *MDH3*, *TPI* and *SKDH*.

3.3. Genotype polymorphism index

The genotype polymorphism index (P_g) for the three age classes of trees of PP and JP is shown in Tables 4 and 5. There was no significant difference in P_g among tree age classes of PP. In JP, P_g was higher in younger trees than in old growth trees.

3.4. Fixation index

The mean value of the fixation index (F) describes whether populations are in Hardy–Weinberg equilibrium (Tables 4

Table 2

Statistically significant differences in frequency of alleles and genotypes (frequencies in parentheses) among the three tree age classes of ponderosa pine (O, old growth; M, mature; S, sapling)

| Alleles | | | | |
|--------------|----------|-----------|----------|------------|
| Locus | Allele | Age class | | Chi-square |
| <i>SKDH1</i> | 1 | O (0.86) | M (0.46) | 7.38 |
| <i>SKDH1</i> | 2 | O (0.00) | M (0.42) | 25.00 |
| <i>SKDH1</i> | 2 | O (0.00) | S (0.43) | 25.65 |
| Genotypes | | | | |
| Locus | Genotype | Age class | | Chi-square |
| <i>MDH3</i> | 12 | O (0.22) | M (0.57) | 4.41 |
| <i>SKDH1</i> | 11 | O (0.78) | M (0.38) | 4.22 |
| <i>SKDH1</i> | 22 | O (0.00) | S (0.35) | 8.36 |

Table 3

Statistically significant differences in frequency of alleles and genotypes among the three tree age classes of Jeffrey pine (O, old growth; M, mature; S, sapling)

| Alleles | | | | |
|--------------|----------|-----------|----------|------------|
| Locus | Allele | Age class | | Chi-square |
| <i>FEST</i> | 2 | O (0.03) | S (0.15) | 3.96 |
| <i>SKDH1</i> | 2 | O (0.00) | S (0.17) | 8.65 |
| <i>SKDH1</i> | 3 | O (0.17) | M (0.00) | 8.55 |
| <i>SKDH1</i> | 3 | O (0.17) | S (0.04) | 3.95 |
| <i>TPI-1</i> | 1 | O (0.74) | M (0.40) | 6.11 |
| <i>TPI-1</i> | 1 | O (0.74) | S (0.42) | 5.35 |
| <i>TPI-1</i> | 2 | O (0.26) | M (0.60) | 8.22 |
| <i>TPI-1</i> | 2 | O (0.26) | S (0.54) | 6.07 |
| Genotypes | | | | |
| Locus | Genotype | Age class | | Chi-square |
| <i>TPI-1</i> | 11 | O (0.63) | M (0.23) | 5.62 |
| <i>TPI-1</i> | 11 | O (0.63) | S (0.27) | 4.39 |

and 5). For PP and JP, an excess of homozygotes was found in the studied groups of trees. The average fixation indices (F) were similar for PP and JP. However, there were differences in the F index among tree age classes for each species. For PP,

Table 4

Observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F) and genotypic polymorphism index (P_g) for the three age classes of ponderosa pine (O, old growth; M, mature; S, sapling)

| Locus | Population | H_e | H_o | F | P_g |
|--------------|------------|--------|--------|---------|--------|
| <i>FEST</i> | O | 0.0000 | 0.0000 | — | 0.0000 |
| | M | 0.0997 | 0.0000 | 1.0000 | 0.0997 |
| | S | 0.1588 | 0.0870 | 0.4524 | 0.2344 |
| <i>GOT1</i> | O | 0.1653 | 0.1818 | −0.1000 | 0.2975 |
| | M | 0.0000 | 0.0000 | — | 0.0000 |
| | S | 0.0000 | 0.0000 | — | 0.0000 |
| <i>MDH3</i> | O | 0.3512 | 0.2727 | 0.2235 | 0.5124 |
| | M | 0.4321 | 0.5263 | −0.2179 | 0.5429 |
| | S | 0.3147 | 0.2174 | 0.3093 | 0.4612 |
| <i>MDH5</i> | O | 0.1684 | 0.1818 | −0.0798 | 0.3099 |
| | M | 0.2368 | 0.2632 | −0.1111 | 0.4211 |
| | S | 0.1238 | 0.1304 | −0.0534 | 0.2344 |
| <i>MNR</i> | O | 0.5539 | 0.4762 | 0.1845 | 0.7392 |
| | M | 0.6080 | 0.4211 | 0.3075 | 0.7590 |
| | S | 0.5992 | 0.4545 | 0.2414 | 0.7727 |
| <i>PGM</i> | O | 0.0444 | 0.0455 | −0.0233 | 0.0868 |
| | M | 0.0512 | 0.0526 | −0.0270 | 0.0997 |
| | S | 0.0000 | 0.0000 | — | 0.0000 |
| <i>SKDH</i> | O | 0.3605 | 0.2727 | 0.2436 | 0.5331 |
| | M | 0.6496 | 0.1579 | 0.7569 | 0.7147 |
| | S | 0.4915 | 0.1739 | 0.6462 | 0.6200 |
| <i>TPI-1</i> | O | 0.3853 | 0.4091 | −0.0617 | 0.5661 |
| | M | 0.4806 | 0.4211 | 0.1239 | 0.6704 |
| | S | 0.3488 | 0.3478 | 0.0027 | 0.5331 |
| Mean value | O | 0.2574 | 0.2300 | 0.1065 | 0.3806 |
| | M | 0.3198 | 0.2303 | 0.2799 | 0.4134 |
| | S | 0.2546 | 0.1764 | 0.3072 | 0.3570 |
| Mean | O + M + S | 0.2772 | 0.2122 | 0.2312 | 0.3837 |

Table 5
Observed heterozygosity (Ho), expected heterozygosity (He), fixation index (F) and genotypic polymorphism index (Pg) for the three age classes of Jeffrey pine (O, old growth; M, mature; S, sapling)

| Locus | Population | He | Ho | F | Pg |
|------------|-------------|--------|--------|---------|--------|
| FEST | O | 0.0260 | 0.0263 | −0.0133 | 0.0512 |
| | M | 0.1189 | 0.1250 | −0.0511 | 0.2257 |
| | S | 0.2604 | 0.2308 | 0.1136 | 0.4112 |
| GOT1 | O | 0.0512 | 0.0526 | −0.0270 | 0.0997 |
| | M | 0.0408 | 0.0417 | −0.0213 | 0.0799 |
| | S | 0.0000 | 0.0000 | — | 0.0000 |
| MDH3 | O | 0.1908 | 0.1053 | 0.4483 | 0.2812 |
| | M | 0.2491 | 0.2083 | 0.1638 | 0.3924 |
| | S | 0.1420 | 0.1538 | −0.0833 | 0.2604 |
| MDH5 | O | 0.1243 | 0.1316 | −0.0585 | 0.2341 |
| | M | 0.0799 | 0.0000 | 1.0000 | 0.0799 |
| | S | 0.0000 | 0.0000 | — | 0.0000 |
| MNR | O | 0.5313 | 0.3889 | 0.2680 | 0.7037 |
| | M | 0.5877 | 0.6250 | −0.0635 | 0.7257 |
| | S | 0.5355 | 0.6154 | −0.1492 | 0.6746 |
| PGM | O | 0.0512 | 0.0526 | −0.0270 | 0.0997 |
| | M | 0.0000 | 0.0000 | — | 0.0000 |
| | S | 0.0000 | 0.0000 | — | 0.0000 |
| SKDH | O | 0.2836 | 0.0789 | 0.7216 | 0.3532 |
| | M | 0.3377 | 0.1250 | 0.6298 | 0.4132 |
| | S | 0.3469 | 0.2692 | 0.2239 | 0.5237 |
| TPI-1 | O | 0.3324 | 0.2105 | 0.3667 | 0.4765 |
| | M | 0.4575 | 0.3750 | 0.1803 | 0.6215 |
| | S | 0.5296 | 0.3077 | 0.4190 | 0.6893 |
| Mean value | O | 0.1989 | 0.1308 | 0.3420 | 0.2874 |
| | M | 0.2339 | 0.1875 | 0.1985 | 0.3173 |
| | S | 0.2268 | 0.1971 | 0.1309 | 0.3199 |
| Mean | (O + M + S) | 0.2199 | 0.1718 | 0.2238 | 0.3082 |

homozygosity level decreased from younger trees to old growth. JP had the opposite response.

3.5. Genetic distances

Comparison of genetic distance indices based on the frequency of alleles (SN) and genotypes (SH) indicated that average genetic distances were two times higher between the tree age classes in PP than in JP (Table 6). Genetic distances between parental and young trees of PP and JP were distinctly higher than the genetic distances between the two species. Comparison of genetic similarities' indices based on SN and SH indicated that in both species, trees in sapling and mature

age classes were the most similar genetically. Comparative SN values ranged between 0.01 and 0.06.

3.6. Level of O₃ injury in the two species and across tree age classes

Individual tree health as determined by canopy health attributes previously shown to be responsive to O₃ exposure exhibited significant differences in the number of live whorls retained (old growth, 2003), chlorotic mottle of 2-yr-old needles (saplings in both 2001 and 2003; mature trees in 2003), and branch diameter (saplings and mature trees in 2003) between PP and JP (Table 7). Individual canopy health attributes differed significantly among tree age classes for percent branch foliated (both species, both years), needle elongation growth (both species in 2001; JP in 2003), and branch elongation and diameter growth (both species, both years). A commonly used index of O₃ injury in yellow pine throughout the western United States did not significantly differ among tree age classes for either species or year (Table 7). However, the higher score in 2003 for both species suggests an overall improved canopy health in that year, better canopy health for parental vs. progeny, and better canopy health for JP vs. PP.

To integrate the response of the individual morphological attributes within a species and tree age class, a detrended coordinate analysis (DCA) was applied to both the 2001 and 2003 data (PC-ORD, 1999). The coefficient of the ordinations was >0.95, using three axes. The tree age classes had different morphological responses in the 2 yr of the study, but it is clear that canopy health attributes of old growth PP and JP differed from that of younger tree age classes (Fig. 4). Old growth PP differed significantly from old growth JP only in eigenvector 3 in 2001. In general, eigenvalues of sapling and mature trees of both species did not significantly differ. The exceptions were only for JP: eigenvector 3 in 2001, and eigenvectors 1 and 2 in 2003. In 2001, the 2nd eigenvector significantly differentiated the two species in both mature and old growth tree age classes, and the 3rd eigenvector significantly differentiated the two species in both old growth and saplings. In 2003, the two species were differentiated significantly only for eigenvector 1 for saplings.

4. Discussion

Adaptation to environmental stress contributes to altered genetic structure of forest tree species (Bergmann and Scholz, 1989). Environmental stress may be sufficiently severe that sensitive individuals are lost in a population and specific genes change in frequency, resulting in changes in the genetic structure of the population (Bergmann and Scholz, 1989). In general, less homozygotic individuals are better adapted to environmentally stressful conditions (Hedrick, 1976, 1978; Lewontin, 1974; Zouros and Foltz, 1987). However, under extreme environmental conditions, a decreased level of heterozygosity may also be found because only special genotypes can survive in such environments (Prus-Glowacki and Godzik, 1991; Prus-Glowacki and Nowak-Bzowy, 1992; Scholz,

Table 6
Genetic similarity coefficients among age classes in both pine species

| | Jeffrey pine | | | Ponderosa pine | | |
|------|--------------|-------|-------|----------------|-------|-------|
| | SN O | SN M | SN S | SN O | SN M | SN S |
| SH O | | 0.963 | 0.973 | | 0.983 | 0.974 |
| SH M | 0.944 | | 0.994 | 0.945 | | 0.985 |
| SH S | 0.945 | 0.985 | | 0.962 | 0.956 | |

SN indicates frequency of alleles and SH indicates frequency of genotypes.

Table 7
Morphological attributes of three tree age classes of ponderosa and Jeffrey pine

| | Ponderosa pine | | | Jeffrey pine | | |
|---------------------------------------|----------------|-------------|------------|----------------|----------------|--------------|
| | Saplings | Mature | Old growth | Saplings | Mature | Old growth |
| Tree age, yr | 17 ± 1 | 86 ± 8 | >200 | 18 ± 1 | 79 ± 6 | >200 |
| Bole diameter, cm | 6 ± 1 | 48 ± 2 | 106 ± 4 | 5 ± 0 | 42 ± 2 | 99 ± 3 |
| FPM score | | | | | | |
| 2001 | 3.3 ± 0.3 | 3.4 ± 0.2 | 3.6 ± 0.2 | 3.6 ± 0.1 | 3.7 ± 0.2 | 4.1 ± 0.2 |
| 2003 | 3.8 ± 0.5 | 4.4 ± 0.4 | 4.3 ± 0.1 | 4.0 ± 0.2 | 4.0 ± 0.2 | 4.2 ± 0.2 |
| No. live whorls | | | | | | |
| 2001 | 4.3 ± 0.3 | 4.2 ± 0.2 | 4.5 ± 0.2 | 4.2 ± 0.2 | 3.9 ± 0.2 | 4.4 ± 0.2 |
| 2003 | 4.9 ± 0.3 | 5.0 ± 0.2 | 5.4 ± 0.2* | 4.8 ± 0.2 | 4.6 ± 0.2 | 4.7 ± 0.2* |
| % Branch foliated | | | | | | |
| 2001 | 63 ± 4a | 69 ± 3a | 56 ± 2b | 62 ± 3(1) | 68 ± 2(1) | 54 ± 2(2) |
| 2003 | 67 ± 5a | 72 ± 2a | 57 ± 3b | 73 ± 3(1) | 72 ± 2(1) | 52 ± 2(2) |
| Chlorotic mottle, %, 2-yr-old needles | | | | | | |
| 2001 | 10 ± 2* | 7 ± 1 | 6 ± 1 | 7 ± 1* | 6 ± 1 | 5 ± 1 |
| 2003 | 11 ± 6a* | 3 ± 0b§ | 3 ± 0b | 4 ± 0* | 5 ± 1§ | 3 ± 0 |
| Chlorotic mottle, %, 4-yr-old needles | | | | | | |
| 2001 | 36 ± 9 | 40 ± 9 | 33 ± 6 | 36 ± 5 | 27 ± 6 | 28 ± 7 |
| 2003 | 21 ± 4 | 16 ± 4 | 21 ± 2 | 16 ± 2 | 26 ± 5 | 30 ± 6 |
| Needle length, mm | | | | | | |
| 2001 | 180 ± 8a | 190 ± 4a | 151 ± 8b | 193 ± 7(1) | 179 ± 6(2) | 151 ± 6(3) |
| 2003 | 181 ± 2 | 176 ± 8 | 152 ± 8 | 202 ± 6(1) | 177 ± 5(2) | 150 ± 5(3) |
| Branch length, mm | | | | | | |
| 2001 | 9 ± 3a | 32 ± 2b | 94 ± 9c | 30 ± 16(1) | 29 ± 2(1) | 75 ± 7(2) |
| 2003 | 25 ± 2a | 24 ± 2a | 52 ± 8b | 27 ± 1(1) | 23 ± 2(2) | 52 ± 7(3) |
| Branch diameter, mm | | | | | | |
| 2001 | 9.2 ± 9.6a | 10.0 ± 0.5a | 7.4 ± 0.5b | 10.7 ± 0.4(1) | 10.1 ± 0.5(1) | 7.4 ± 0.5(2) |
| 2003 | 9.6 ± 0.3a§ | 8.4 ± 0.4b* | 7.4 ± 0.3c | 11.0 ± 0.3(1)§ | 10.3 ± 0.3(1)* | 7.6 ± 0.3(2) |

Except for chlorotic mottle, mean ± 1 S.E. were reported for 2-yr-old tissue. Within a year, within a species, differing letters (ponderosa pine) or numbers (Jeffrey pine) denote statistical significance. Comparisons between years were not made due to differences in sample size from 2001 to 2003 (not all individuals relocated in 2003). Statistical significance is reported at the $P = 0.05$ level using paired t -tests (SAS, 2003). § and * denote significant differences between values within the row of data in Table 7.

1990). Examples of both adaptation strategies have been reported for conifers in response to heavy metals or excessive sulphur dioxide exposure (Mejnartowicz, 1983; Scholz and Bergmann, 1984; Geburek et al., 1987; Prus-Glowacki and Nowak-Bzowy, 1989, 1992; Prus-Glowacki and Godzik, 1991; Prus-Glowacki et al., 2006). Decreased genetic variation from the elimination of sensitive individuals constitutes an erosion of the gene pool, and can reduce the adaptability of the population to local conditions as well as future changes in the environment.

Although not generally believed to be strong enough to induce changes in population genetic structure, the genetic basis of O_3 tolerance was first established in Bel W-3 tobacco (Taylor, 1968). Subsequently, the genetic basis of O_3 tolerance has been reported for a number of species, including: potato (*Solanum tuberosum* L., DeVos et al., 1982), bladder campion (*Silene cucubalus*, Ernst et al., 1985), and onion (*Allium cepa* L., Engel and Gabelman, 1989). Perhaps the best example of the genetic basis of O_3 tolerance in a tree species was established for aspen (*Populus tremuloides* Michx., Karnosky, 1977), with subsequent field verification (Berrang et al., 1989). Most recently, O_3 resistant mature ponderosa pine were found to be more heterozygous than O_3 sensitive individuals in a forest

exposed consistently to long term, high O_3 exposure (Staszak et al., 2004).

In our study, the mean number of alleles, as well as the mean number of genotypes per locus, exhibited the same tendency in both species: the number of alleles and genotypes increased with increasing age of trees. Wright's index (F) indicated an excess of homozygotes in both species but in PP, homozygosity was higher in the two younger tree age classes relative to old growth trees, and the observed heterozygosity was higher in old growth trees by 24%. In JP, homozygosity was higher in the old growth tree age class relative to saplings, and the observed heterozygosity was higher in saplings by 34%. These trends were reflected in the fixation index for both species. Considering that PP is more sensitive to oxidant pollution than JP (Miller et al., 1983), it is reasonable that the two species exhibit different adaptive responses to oxidative stress. More heterozygotic saplings relative to parental trees would be expected if significant environmental stress were imposed on younger tree age classes, selecting for survival of more heterozygotic seedlings, through greater success of progeny resulting from outbreeding, not from local parental crosses (e.g., JP). The average genetic distance indices (SN and SH) between tree age classes in PP and JP were similar

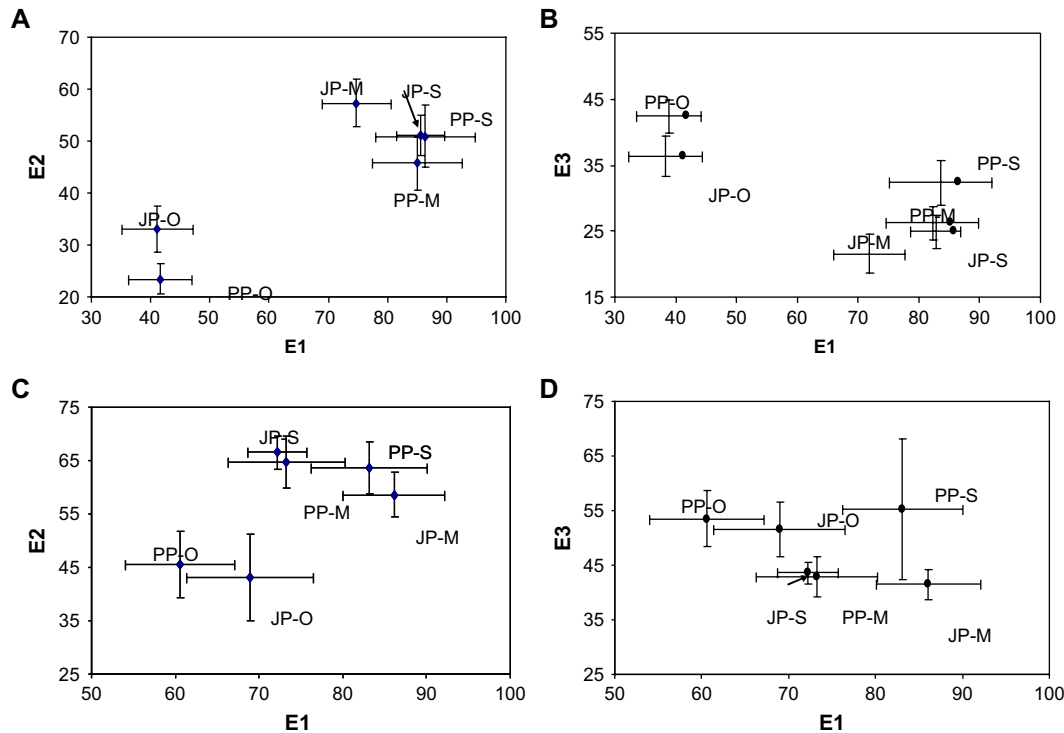


Fig. 4. Detrended correspondence analysis (DCA) for 2001 (A, eigenvector 1 vs. 2; B, eigenvector 1 vs. 3) and 2003 (C, 1 vs. 2; D, 1 vs. 3). The points and error bars (mean \pm 1 S.E. for eigenvalues) are identified by species (PP, ponderosa pine; or JP, Jeffrey pine) and by tree age class (O, old growth; M, mature; S, sapling).

suggesting similar adaptations. In an earlier publication we demonstrated that O_3 -tolerant individuals of PP exhibited higher heterozygosity than O_3 -sensitive individuals (Staszak et al., 2004). Judging from genetic similarity indices, the most pronounced changes in genetic structure occurred between younger (sapling and mature) tree age classes and the old growth trees. Assuming that O_3 exposure did not increase significantly until after 1945 (see Fig. 3), these data suggest that mature trees also experienced sufficient environmental stress (from age 30 to 80 yr) to influence genetic structure.

Differences in frequency of alleles and genotypes, mean number of alleles and genotypes per locus, level of heterozygosity (H_o) and genotypic polymorphism (P_g) between young and older tree age classes suggest that changes in the genetic structure of both PP and JP have occurred. The greatest differences in heterozygosity were found for loci *SKDH*, *FEST*, *MNR*, *MDH-C*, and *TPI*. Most of these enzyme loci except for *TPI* have been previously identified as suitable markers for studying changes in the genetic structure caused by pollution (Bergmann and Scholz, 1985, 1987, 1989; Geburek et al., 1986; Prus-Glowacki and Godzik, 1995; Prus-Glowacki et al., 1999).

Although we have inferred changes in O_3 concentrations over the lifetimes of the three tree age classes, other environmental stressors may have had an effect on the genetic structure of yellow pine populations in Sequoia National Park. These stressors include periodic severe drought stress, pathogen and insect infestations, fire disturbance, land use changes, excess nitrogen deposition, and long term increases in temperature. Based on a 70-yr precipitation record in Sequoia

National Park, there were two periods which would have induced extreme drought stress in ponderosa pine, inferred from physiological measurements made in southern California on PP (Grulke et al., in press): 1959–1961 (averaging 54% of the long term average annual precipitation record), and 1987–1990 (averaging 62% of the long term average; Fig. 5). These multiple-year droughts resulted in elevated mortality in yellow pine in the park (Warner, 1987). It is likely that the saplings in this study established during years of largely above-average precipitation 1978–1986, just prior to the 4-yr, late 1980s drought. The saplings would likely have experienced the greatest drought stress because they were \sim 10-yr-old (vs. \sim 60-yr-old for mature trees) at that time.

There was an historic bark beetle outbreak (prior to precipitation records for the region) in 1913–1918 affecting yellow pine (Miller 1940, as seen Warner, 1987) (reported epidemic bark beetle infestations are indicated with arrows on Fig. 5). Although the saplings were cored at the base of the stem, the mature trees were cored at 1.4 m, and so were at least that height at the time of the bark beetle infestation in the mid 1900s. It is likely that the mature trees in this study established during years of largely above-average precipitation 1900–1910. The current mature trees thus also likely experienced drought stress early in their establishment.

Fire records in the park have been tracked since 1921 (Caprio and Graber, 2000). Prescribed fire affected one-third of the mature and old growth trees in this study in 1972 (www.nps.gov/archive/seki/fire/fire_map.htm), prior to the establishment of one-third of the saplings in this study. Forest stands are not managed except in the immediate vicinity of

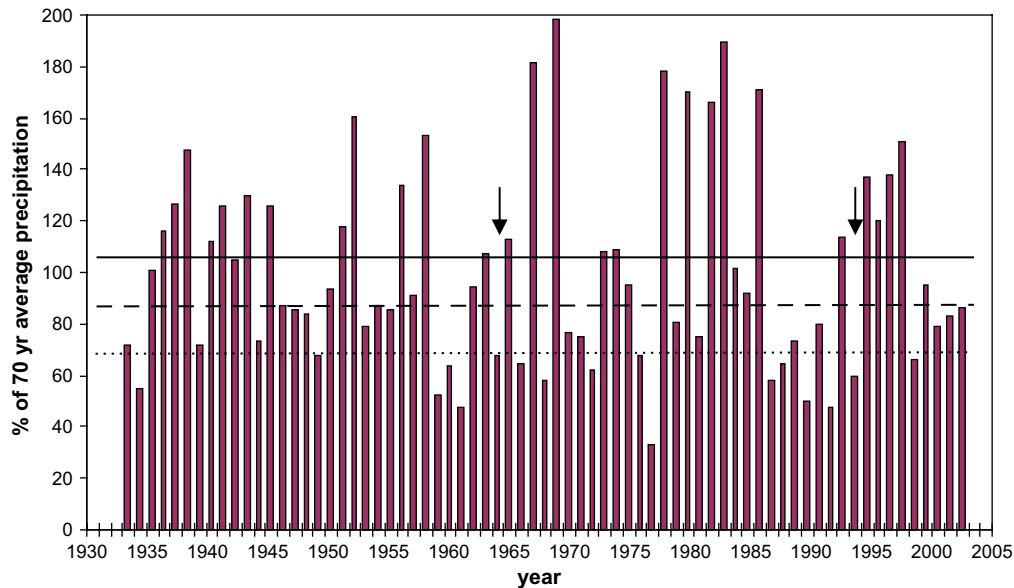


Fig. 5. Precipitation record for Giant Forest (until 1968) and Lodgepole (after 1968) in Sequoia National Park. Dashed line indicates physiologically defined moderate drought stress and dotted line indicates extreme drought stress for ponderosa pine (Grulke, 1999 for moderate drought stress and Grulke, unpublished data for extreme drought stress). Arrows indicate periods of expected high bark beetle attacks based on level of physiological drought stress (Grulke et al., in press).

developed areas (Warner, 1987). Near Sunset Rocks, a campground was abandoned in the early 1970s. Approximately one-quarter of the trees (in all age classes) established outside of the campground. Otherwise, land use has changed little since the park was established in 1890. Although nitrogen deposition accompanies O_3 transport into the park, total annual deposition rates are low ($\sim 6 \text{ kg ha}^{-1} \text{ yr}^{-1}$; Fenn et al., 2003).

Temperature records exist from 1931 to present, although the location of the station was changed from Giant Forest to Lodgepole (6 km north) in November 1968, a site of greater cold air drainage. Compared to the 70-yr record, daily minimum and maximum air temperatures were 3.7 and 0.5 °C greater than the average for the period prior to and including 1968. After 1968, daily minimum and maximum air temperatures were 6.5 and 2.7 °C lower than that of the 70-yr record. Because of the shift in station location, we cannot rule out the potential role of temperature in our observations. Higher temperature, increased drought stress, and increased O_3 exposure can all contribute to increased oxidative stress in plants (Elstner and Osswald, 1994).

The level of drought experienced modifies the expression of foliar O_3 symptoms (Grulke, 2003). In 2001 and 2003 when canopy O_3 injury was assessed, the total annual precipitation was similar (80% of the 70-yr record, Fig. 5), but 2001 was preceded by an average precipitation year (95%, 2000) and a below-average precipitation year (65%, 1999). The canopy assessment in 2003 was preceded by two below-average precipitation years of 80%. The FPM score suggests that canopy health was improved in 2003 relative to 2001, that old growth trees had greater canopy health than saplings, and that canopy health of PP was greater than JP in 2003 but poorer than JP in 2001 (Table 5). In this study, the FPM score was not sensitive enough to elucidate statistically significant differences among tree age classes or between the two species. Using a more

quantitative, multivariate assessment of the attributes of canopy health, an ordination demonstrated significant differences among the tree age classes (Fig. 4). The strongest differences were between the old growth trees and the younger tree age classes.

Foliar retention in yellow pine is highly responsive to O_3 exposure, drought stress, and nitrogen deposition (Grulke and Balduman, 1999). Old growth trees had significantly less within-whorl needle retention (as indicated by lower % branch foliated; Table 5) in both species, in both years. Older needles within a whorl, and more O_3 -symptomatic needles are sloughed prematurely (see % branch foliated), which contributes to an apparent decrease in chlorotic mottle in old growth trees (Grulke, 2003), especially when combined with drought stress. Chlorotic mottle (definitive response to O_3 exposure, Miller et al., 1996) of 2-yr-old needles was significantly greater in PP saplings than in JP saplings in both years (Table 5), and in mature PP in 2003. The level of chlorotic mottle can decrease within a needle age class (the whorl) when the more O_3 -injured needles are lost (e.g., with O_3 and/or drought). A reduction in within-whorl needle retention would explain the differences observed because significantly more needles were retained within-whorl in saplings and mature trees. Chlorotic mottle of 4-yr-old needles mirrored that of the FPM score: PP had greater chlorotic mottle than JP in 2001 but less chlorotic mottle than JP in 2003.

Differences in frequency of alleles and genotypes, mean number of alleles and genotypes per locus, level of heterozygosity (Ho) and genotypic polymorphism (Pg) between saplings and old growth trees suggest that changes in the genetic structure of both PP and JP populations have occurred. We selected isozyme markers associated with O_3 tolerance. Because both drought and O_3 induce oxidative stress, both stressors could have contributed to the observed shifts in

genetic structure. Our data suggest that an increase in pollution over the last ~50 yr, and episodic drought stress may have affected the genetic structure of two species of yellow pine in Sequoia National Park but to a greater degree in PP, a species more sensitive to oxidant pollution.

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